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(21) International Application Number: PCT/US96/13626 (22) International Filing Date: 26 August 1996 (26.08.96) (30) Priority Data: 08/519,039 24 August 1995 (24.08.95) US (60) Parent Application or Grant (63) Related by Continuation US 08/519,039 (CIP) Filed on 24 August 1995 (24.08.95) (71) Applicant (for all designated States except US): THEOBALD SMITH RESEARCH INSTITUTE, INC. [US/US]; 18 Tremont Street, Boston, MA 02108 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): SU, Xing [CN/US]; 221 Lexington Street, Belmont, MA 02178 (US). (74) Agents: YANKWICH, Leon, R. et al.; Banner & Witcoff, Ltd., Ten South Wacker Drive, Chicago, IL 60606 (US).		(81) Designated States: AU, CA, CN, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: METHOD AND APPARATUS FOR ISOLATING NUCLEIC ACID (57) Abstract The invention features a method of isolating nucleic acid in a substantially purified form, including the steps of: a) contacting a biological sample which contains nucleic acid with a matrix comprising a solid hydrophilic organic polymer without an effective positive charge under conditions which permit the nucleic acid to bind to the matrix; and b) recovering nucleic acid from the matrix.		

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METHOD AND APPARATUS FOR ISOLATING NUCLEIC ACID
FIELD OF THE INVENTION

The invention relates to nucleic acid isolation, and more particularly to compositions and methods useful for isolation of nucleic acids.

BACKGROUND OF THE INVENTION

Nucleic acid purification from biological sources or post-enzymatic reactions is frequently a primary step in molecular biology studies and diagnostic tests. Many techniques have been developed to isolate DNA and RNA, for example, phenol extraction, alcohol precipitation, density gradients, dialysis, ion exchange, electroelution, silica binding, membrane filtration, and column filtration.

U.S. Patent No. 5,346,994 to Chomczynski discloses a liquid nucleic acid isolation method employing a reagent mixture of phenol, chaotropic salts and stabilizers. The procedure involves cell lysis and separation of DNA, RNA and proteins in different phases by centrifugation.

U.S. Patent Nos. 5,187,083 and 5,234,824 to Mullis disclose DNA isolation methods which rely on physical trapping of high molecular weight DNA on membranes, such as cellulose acetate filters. These methods are designed for large DNA and are not effective for generally isolating DNAs of any size or molecular weight.

A common approach to isolating and purifying nucleic acids involves binding of the negatively charged phosphodiester backbone of the nucleic acid to a positively charged polymer by electrostatic interactions (ion exchange).

1 U.S. Patent No. 4,935,342 to Seligson et al. discloses a
2 nucleic acid isolation method in which positively charged
3 anion exchange materials are utilized. The nucleic acids are
4 released from the matrices in solutions of high ionic strength
5 (i.e., high salt concentration). However, removal of the salt
6 is often necessary before the nucleic acids may be utilized
7 further.

8 U.S. Patent No. 5,329,000 to Woodard et al. discloses a
9 method of isolating DNA using a silicon tetrahydrazide matrix.
10 Similarly, U.S. Patent No. 5,342,931 to Woodard et al.,
11 discloses a DNA isolation method using a matrix of hydrated
12 silica. In both cases, DNA binds to the inorganic matrix and
13 is released in heated water.

14 U.S. Patent No. 4,923,978 discloses a method of isolating
15 nucleic acids using hydroxylated matrices in a column. In
16 this approach, proteins are subtracted from the biological
17 sample via adsorption onto the matrix, and nucleic acids flow
18 through the matrix. However, because this procedure is
19 subtractive, further purification and concentration of the
20 nucleic acid is often required.

21 One object of the invention is to isolate nucleic acid
22 from a biological sample in a simple, fast, and efficient
23 process that avoids heating the nucleic acid to obtain
24 elution. Another object is to provide a method which avoids
25 high speed centrifugation and phase separation to isolate a
26 nucleic acid. Another object is to provide for highly
27 quantitative recovery of nucleic acid regardless of its
28 molecular weight. Another object of the invention is to

1 isolate nucleic acid from a number of samples simultaneously,
2 thus saving time and effort and providing for subsequent
3 simultaneous processing of the samples. Yet another object of
4 the invention is to provide for simultaneous processing and
5 recovery of even small amounts of nucleic acids from multiple
6 samples. Another object of the invention is to avoid the risk
7 of loss of an isolated nucleic acid by providing a nucleic
8 acid preparation which does not require further concentration
9 from a large volume or does not require further purification.
10 Yet another object of the invention is to provide for high
11 yield recovery of nucleic acid within a broad size range.
12 Another object is to provide a method of nucleic acid
13 isolation that is environmentally friendly, i.e., that avoids
14 the required use of toxic chemicals, corrosive agents or
15 chaotropic salts.

16 SUMMARY OF THE INVENTION

17 The invention is based on a method and apparatus for
18 nucleic acid isolation. The invention utilizes the properties
19 of aggregated nucleic acids to isolate and purify nucleic
20 acids from contaminants such as other cellular components.
21 The invention is based on the discovery that aggregated
22 nucleic acid is capable of binding reversibly to a solid,
23 hydrophilic organic matrix without an effective positive
24 charge.

25 The invention therefore encompasses a method of isolating
26 nucleic acid in a substantially purified form, the method
27 comprising the steps of: a) contacting a biological sample

1 comprising aggregated nucleic acid with a matrix other than
2 cellulose or a cellulose matrix suspension under conditions
3 which permit nucleic acid in the sample to reversibly bind to
4 the matrix, the matrix comprising a solid hydrophilic organic
5 polymer without an effective positive charge; and b)
6 recovering nucleic acid from the matrix.

7 Preferably, in the recovering step, the nucleic acid is
8 eluted from the matrix in substantially purified and
9 concentrated form without heat; that is, preferably
10 substantially at room temperature or at about 80°F or less,
11 i.e., in the range of about 50-80°F or about 60-80°F. The
12 recovery step may also comprise dissociation and/or
13 solubilization of the nucleic acid aggregates in water or a
14 low salt buffer.

15 As used herein, the terms "contacting a biological sample
16 with a matrix" refers to any means known in the art of bringing
17 the nucleic acid into physical contact with a matrix as
18 described herein.

19 As used herein, "biological sample" refers to a sample of
20 biological or biochemical origin; "substantially purified"
21 refers to recovery of nucleic acid which is at least 80% and
22 preferably 90-95% purified with respect to removal of a
23 contaminant, e.g., cellular components such as protein, lipid
24 or salt; thus, the term "substantially purified" generally
25 refers to separation of a majority of cellular proteins or
26 reaction contaminants from the sample, so that compounds
27 capable of interfering with the subsequent use of the isolated
28 nucleic acid are removed; "bind to" refers to reversible

1 binding via weak forces such as Van der Waals interactions,
2 and does not refer to electrostatic interactions, affinity
3 binding or physical trapping. As used herein, "Van der Waals
4 interactions" refer to the weak forces between atoms and
5 molecules due to induced or instantaneous dipole movements,
6 which may allow packing together of organic compounds such as
7 nucleic acid and other hydrophilic organic materials (matrix).
8 "Electrostatic interactions" refer to positive (+) and
9 negative (-) charge attractions; "affinity binding" refers to
10 the sequence- or conformation-specific and directional binding
11 between a molecule and its complementary molecule; and
12 "physical trapping" refers to retaining of nucleic acid based
13 on the relatively large size of the nucleic acid and small
14 size of holes in the matrix.

15 As used herein, "without an effective positive charge"
16 refers to a matrix having a net (i.e., overall) neutral or a
17 net negative charge, or a matrix which does not possess
18 sufficient positive charge to attract, bind or retain a
19 measurable or detectable (i.e., by optical density measurement
20 at 260) amount of nucleic acid in low ionic strength buffer
21 (i.e., <10 mM of Na⁺, pH 5-10). A net charge is calculated by
22 adding together the positive and negative charges in the
23 repeating units of the polymer that constitute the matrix.
24 Thus, a matrix without an effective positive charge is not
25 suitable for use as an ion exchange material for nucleic acid
26 purification by conventional methods.

27 The terms "aggregate" and "aggregation" refer to the
28 tendency of large macromolecules, such as nucleic acids, to

1 combine into clusters or clumps. Thus, nucleic acids are
2 considered "aggregated" when they precipitate or separate from
3 the dissolved or soluble state. A nucleic acid is considered
4 aggregated for the purposes of the invention when essentially
5 no nucleic acid (i.e., less than 10% and preferably less than
6 2% of the nucleic acid present prior to aggregation) remains
7 in solution upon collection of the precipitated nucleic acid
8 by high speed centrifugation, e.g., in a standard microfuge at
9 10,000 rpm, for approximately 10 min. In order for binding to
10 the matrix to be effective, the nucleic acid must be in
11 aggregated form. However, the aggregation step need not be
12 performed prior to the matrix-contacting step; that is, non-
13 aggregated nucleic acid may be mixed with the matrix and
14 aggregation effected thereafter in the mixture.

15 The term "hydrophilic organic polymer" refers to a
16 polymer made of repeating units of organic compounds, i.e.,
17 natural and synthetic forms thereof. The polymer is insoluble
18 in water and alcohol (at <50 degrees C.) and has sufficient
19 amounts of polar groups accessible to water molecules such
20 that water can be retained and absorbed by the matrix.
21 Preferably, the matrix comprises a polymer in which
22 substantially all of the repeat units include polar, uncharged
23 groups at neutral pH. Polar groups are those which have
24 abilities to form hydrogen bonds with water molecules.

25 Preferably, the matrix is a hydrophilic naturally
26 occurring compound or a synthetic organic compound or a
27 hydrophilic derivative of a hydrophobic compound. Hydrophilic
28 derivatives of a hydrophobic compound (backbone), such as a

1 plastic material, can be obtained by modifying the backbone
2 with the addition of polar groups, including hydroxyl groups
3 (OH), carboxyl groups (COOH), amino groups (NH₂), groups which
4 are neutral at pH > 7, and thiol groups (SH), such that the
5 surfaces of fibrous and particulate matrices comprising this
6 polymer then possess hydrophilic properties.

7 A hydrophilic compound or a modifiable hydrophobic
8 backbone is selected from the group of polysaccharides
9 including cellulose, rayon, cellulose acetate, cellulose
10 triacetate, chitin and agarose. A hydrophilic compound also
11 may be selected from the group of protein/polypeptides
12 including leather, silk and wool. It can also be selected
13 from synthetic gels including polyacrylamide, hydrogel (i.e.,
14 copolymer of poly(vinyl alcohol) and collagen). It may also
15 be selected from the group of synthetic fibers including
16 polyamides (nylon), polyesters, polyacrylonitrile (acrylic),
17 polyurethane (spandex). It can also be selected from the
18 group of synthetic plastics including polycarbonate, phenol-
19 formaldehyde resins, polysulfide, poly(vinyl butyryl),
20 poly(vinyl chloride), poly(vinylidene chloride),
21 poly(ethylene), and polystyrene.

22 Fibrous or particulate forms of a polymer can be readily
23 prepared by mechanical means well-known in the art. "Fibrous"
24 refers to fibers of e.g., 1 micrometer - 10 micrometers and
25 as long as 100, 1,000 or 10,000 micrometers; and "particulate"
26 refers to particles of e.g., about 1 micron to 5 microns, or
27 even as large as 10, 50, or 100 microns in diameter. Some
28 examples of fibrous cellulose currently on the market include

1 Sigma CF11 cellulose catalog # C6288. An example of
2 particulate cellulose currently on the market is microgranular
3 cellulose Sigma C-6413 and product number CM1000, Megacell,
4 sold by Cortex Biochemicals, San Leandro CA. Particulate
5 cellulose includes any microgranular substance coated with
6 cellulose, e.g., cellulose-coated magnetic beads.

7 Preferably, the aggregated nucleic acid binds to a matrix
8 suspension.

9 As used herein, "matrix suspension" refers to an insoluble
10 matrix immersed in a liquid such that free-floating pieces of
11 the matrix can move freely relative to the container and
12 relative to other free-floating pieces of matrix when the
13 container is shaken or when the liquid is stirred. One
14 example of a matrix suspension is cellulose fibers immersed in
15 liquid at a density such that the liquid appears visually
16 turbid when the container is shaken, and appears to clear when
17 the cellulose fibers settle to the bottom of a stationary
18 container. That is, when the container is shaken, the
19 cellulose fibers move freely relative to the container and to
20 each other. A matrix suspension does not refer to a
21 suspension of a support material having an immobilized matrix
22 attached thereto. However, the invention may encompass a
23 suspension of sets of immobilized cellulose fibers, each set
24 being cellulose fibers attached to each other indirectly via a
25 support, and each set moving freely relative to other sets
26 upon movement of the immersing liquid. That is, when shaken,
27 all sets move freely relative to the container and each other,
28 and cellulose fibers within a set have a fixed range of

1 movement relative to each other.

2 In contrast, a matrix which is not in suspension is a
3 solid material packed into a container, for example, into a
4 cylinder or conical-shaped container that may remain at both
5 ends such that only the liquid, and not the solid materials,
6 moves freely through the container. A typical non-suspension
7 cellulose matrix is known in the art as a "column" containing a
8 packed cellulose matrix. This type of column can retain
9 nucleic acid by physical entrapment within the cellulose
10 matrix. A column containing a packed matrix is not to be
11 confused with a column containing a suspension matrix. The
12 key to distinguishing a matrix suspension from a non-matrix
13 suspension is the freedom of movement of the matrix relative
14 to other particles of matrix or relative to the container.

15 A "matrix-collection" device also is useful according to
16 the invention and refers to a cylinder or conical-shaped
17 container that is open at both ends to liquid movement and fit
18 with a barrier at one end to prevent solid materials from
19 passing through.

20 In preferred embodiments, the method further includes the
21 initial step of contacting the biological sample with a buffer
22 under conditions to solubilize the nucleic acid, i.e., to
23 dissolve the nucleic acid. These conditions include
24 resuspension of the nucleic acid in an aqueous (e.g. Tris-
25 EDTA) buffer, contacting the biological sample in a detergent
26 buffer with a proteolytic enzyme under conditions sufficient
27 to subject the sample to proteolysis and release of nucleic
28 acid, contacting the biological sample with chaotropic agents,

1 or other methods known in the art to release nucleic acids
2 from cellular components into solution.

3 The method further includes the step of aggregating the
4 nucleic acid, wherein the nucleic acid is aggregated by
5 contacting the biological sample with a precipitant selected
6 from the group consisting of organic solvents, soluble organic
7 polymers and salts, (and combinations thereof) wherein the
8 organic solvent may be any one of isopropanol, ethanol,
9 acetone, and organic polymers including but not limited to
10 polyethylene glycol (PEG), and wherein the salt may include
11 but is not limited to NaCl and LiCl. Co-precipitants, such as
12 glycogen, also may be used to facilitate the precipitation of
13 nucleic acid present in only small quantities; for example, in
14 the form of heteroaggregates. The presence of a co-
15 precipitant is not required according to the invention, but
16 serves to increase the efficiency of aggregate formation.

17 Small molecules and digested proteins do not bind to the
18 matrices and thus may be separated from the nucleic acid by
19 washing the adsorbed, aggregated nucleic acid. Therefore, the
20 method may also include a washing step using solutions that
21 contain a precipitant at a concentration sufficient to
22 maintain a nucleic acid in aggregated form.

23 The invention also includes an apparatus for isolating
24 nucleic acid in a substantially purified form from multiple
25 biological samples simultaneously, the apparatus comprising:
26 plural housings for isolation of nucleic acid from plural
27 biological samples, wherein each housing comprises an inlet
28 and an outlet and defines a flowpath for flow of a biological

1 sample therethrough, the flowpath comprising a matrix
2 comprising a solid hydrophilic organic polymer without a net
3 positive charge, and support means for holding plural housings
4 in place such that nucleic acid in plural biological samples
5 may be handled and isolated simultaneously.

6 Preferably, each housing of the apparatus further
7 includes a barrier means to allow flow of liquid along the
8 flowpath and through the housing outlet, but to prevent the
9 matrix from exiting the housing via the outlet. Most
10 preferably, the housing comprises a lower portion leading to
11 the outlet and the barrier is positioned within the lower
12 portion of the housing. The apparatus may also include means
13 for connecting the plural housings to a vacuum, magnetic or
14 pressure source. The barrier may be any material which
15 prevents the matrix from exiting the housing but which allows
16 liquid comprising nucleic acid to flow through and exit the
17 housing, for example, a mesh screen, cotton fibers, synthetic
18 fibers, tissue paper, or siliconized glass fibers. The
19 apparatus may also include a collection tray for
20 simultaneously collecting waste or plural nucleic acid
21 samples. In a preferred embodiment, the apparatus may be
22 subjected to vacuum force, pressure force or low
23 centrifugation force. In another embodiment, the apparatus is
24 constructed such that it does not allow for cross-
25 contamination of the samples.

26 The invention also encompasses an apparatus comprising a
27 matrix-coated surface, for example, a flat surface onto which
28 a sufficient amount of cellulose is coated so as to permit

1 binding of aggregated nucleic acid to the surface, e.g., a
2 cellulose-coated plastic or glass column or microtiter dish.

3 The method and apparatus of the present invention permits
4 the isolation of nucleic acid having essentially any molecular
5 weight or form (i.e., circular, linear, etc.) in a rapid and
6 high-yield manner. The apparatus allows for such isolation
7 from plural samples simultaneously.

8 The nucleic acid isolated as described herein may be of
9 any molecular weight and in single-stranded or double-stranded
10 form; i.e., small oligonucleotides such as 10 - 50 bases in
11 length, small nucleic acid fragments of, for example, 100
12 bases - 500 bases in length, or relatively longer fragments of
13 1000 bases - 10,000 bases in length. Alternatively, high
14 molecular weight nucleic acid, e.g., 50 kb-500 kb may be
15 isolated as described herein. Preferably, a nucleic acid
16 isolated according to the invention will be in the range of 50
17 bases to 500 kilobases.

18 The nucleic acid sample applied to the matrix according
19 to the methods described above may be in any convenient
20 volume. Where large-scale isolation is contemplated, the
21 applied volume may be correspondingly large, e.g., 1 liter,
22 500 ml, 100 ml, 50 ml, etc. Alternatively, where other than
23 large-scale isolation is contemplated, a correspondingly
24 smaller volume may be applied to the matrix, e.g., less than
25 50 ml, more preferably, less than 5 ml, less than 500 μ l;
26 e.g., 1-100 μ l.

27 The volume of nucleic acid isolated according to the
28 invention may be in any selected volume which is sufficient to

1 saturate the matrix. For example, for nucleic acid isolation
2 on a large-scale, the volume of isolated nucleic acid may be
3 correspondingly large, e.g., 1 - 100 ml, as described above
4 for the applied volume. Alternatively, the isolated nucleic
5 acid may be recovered in a smaller volume, e.g., less than 500
6 μ l, 250 μ l, 100 μ l; e.g., 1-50 μ l.

7 The nucleic acid applied to the matrix, as described
8 herein, may be any amount, that amount being determined by the
9 amount of matrix. Preferably, the amount of nucleic acid (and
10 plus co-precipitant, if desired) applied to the matrix is less
11 than the dried weight of the matrix, typically in the range of
12 1/10,000 to 1/10 (weight nucleic acid/matrix). The amount of
13 nucleic acid applied to the matrix may be as much as 100 gm or
14 as little as a few molecules. Preferably, the amount of
15 nucleic acid applied to the matrix is less than a total of 100
16 mg, more preferably in the range of 10 mg-0.1 ng, and most
17 preferably, in the range of 500 μ g-1 ng. The nucleic acid
18 isolated from the matrix will generally be in an amount which
19 is about 90% or more than the amount of nucleic acid applied
20 to the matrix.

21 The invention is particularly useful in procedures
22 wherein large numbers of samples are handled simultaneously,
23 for example, in newborn screening, where as many as 4-5
24 million samples of newborn blood nationwide are analyzed
25 annually. Molecular screening of newborns is still in its
26 infancy and large scale screening is still difficult, mainly
27 due to lack of a suitable nucleic acid purification method.

28 The invention provides for easy simultaneous recovery of

1 plural nucleic acid samples. In addition, because the
2 inventive methods and devices do not allow for leaking between
3 sample chambers, the danger of cross-contamination of samples
4 is significantly reduced throughout the isolation procedure,
5 i.e., before, during and after isolation of the sample from
6 the sample matrix. These advantages are especially important
7 for uses contemplated in the invention, i.e., where hundreds
8 of individual samples, e.g., 100, 200-500, or thousands, e.g.,
9 1000, 2000, 3000, 4000-6000, or even up to ten thousand or
10 one-hundred thousand individual samples are analyzed
11 simultaneously.

12 The invention thus provides for high yield recovery of
13 relatively pure nucleic acid molecules from a biological
14 sample. The nucleic acid may be recovered efficiently from a
15 number of samples simultaneously, thus saving time and effort
16 and providing for subsequent simultaneous processing or
17 analysis of numerous nucleic acid samples, if desired. Any
18 number of samples may be subjected to isolation simultaneously
19 according to the invention, e.g., a single sample, two
20 samples, tens of samples, 100's and even thousands of samples
21 are conveniently isolated using the devices and methods
22 disclosed herein. The invention thus provides for isolation
23 of nucleic acids from hundreds or thousands of samples
24 routinely in an efficient and safe manner. The number of
25 simultaneously isolated samples is only limited by the number
26 of sample chambers which are present in a single format. A
27 format may include a single planar surface containing numerous
28 individual sample chambers or it may include multiple

1 surfaces; the latter format would include multiple stacked
2 surfaces or multiple side-by-side surfaces.

3 Nucleic acids may be selectively recovered from impure
4 samples such as body fluids, cells, tissues or other types of
5 biological samples according to the invention. Exceedingly
6 small amounts of nucleic acid molecules may be simultaneously
7 and quantitatively recovered according to the invention. For
8 example, 80-90% of the small amount of DNA or RNA present in
9 the dried blood spot samples that are routinely assayed in
10 newborn screening (e.g., equivalent to 15 μ l newborn blood or
11 about 0.4 μ g DNA) can be recovered. The yield of recovery is
12 mainly dependent on the quality of the dried blood sample
13 rather than the procedure itself. Because the invention
14 provides a nucleic acid preparation that does not require
15 further concentration from a large volume, the invention
16 avoids risk of loss of the isolated nucleic acid.

17 Nucleic acids isolated according to the invention, will
18 be useful, for example, in assays for detection of the
19 presence of a particular nucleic acid sequence in a sample.
20 Such assays are important in the prediction and diagnosis of
21 disease, forensic medicine, epidemiology and public health.
22 For example, isolated DNA may be subjected to hybridization
23 and/or amplification to detect the presence of an infectious
24 virus or a mutant gene in an individual, allowing
25 determination of the probability that the individual will
26 suffer from a disease of infectious or genetic origin. The
27 ability to detect an infectious virus or a mutation in one
28 sample among the hundreds or thousands of samples being

1 screened takes on substantial importance in the early
2 diagnosis or epidemiology of an at-risk population for
3 disease, e.g., the early detection of HIV infection, cancer or
4 susceptibility to cancer, or in the screening of newborns for
5 diseases, where early detection may be instrumental in
6 diagnosis and treatment.

7 In addition, the method can also be used in basic
8 research labs to isolate nucleic acid from cultured cells or
9 biochemical reactions. The purified nucleic acid can be used
10 for enzymatic modification such as restriction enzyme
11 digestion, sequencing and amplification.

12 Further objects and advantages of the invention will be
13 apparent in light of the following description and the claims.

14 BRIEF DESCRIPTION OF THE FIGURES

15 Before describing the invention in detail, the drawings
16 will be briefly described.

17 FIG. 1 is a diagram of an embodiment of the invention
18 employing a sample collection tray; and

19 FIG. 2 is a diagram of another embodiment of the
20 invention adapted for connection to a vacuum source.

21 DETAILED DESCRIPTION OF THE INVENTION

22 The invention encompasses a method and apparatus for
23 nucleic acid isolation and concentration, and takes advantage
24 of the discovery that aggregated nucleic acid binds reversibly
25 to a solid, hydrophilic organic matrix without an effective
26 positive charge.

1 The invention utilizes the properties of aggregated
2 nucleic acid to isolate and separate nucleic acid from other
3 biochemical or cellular components such as heparin, which
4 tends to inhibit sensitive enzymatic or chemical reactions
5 such as PCR. Nucleic acid can be released in aqueous buffer
6 from cells or tissues by essentially any known method, such as
7 mechanical disruption, sonication, detergent solubilization,
8 treatment with chaotropic agents, and the like. Once released
9 from cells or tissues and separated from insoluble materials,
10 nucleic acid in solution is allowed to form aggregates in the
11 presence of precipitants.

12 According to the invention, a nucleic acid aggregate is
13 contacted with a solid, hydrophilic organic matrix without an
14 effective positive charge under conditions and for a time
15 sufficient to allow it to bind reversibly to the matrix. If
16 desired, the matrix-nucleic acid complex may be washed to
17 remove contaminants, then dissociated and/or solubilized, and
18 nucleic acid recovered in water or low salt buffer without
19 heat. The method of the present invention permits the
20 investigator or technician to isolate nucleic acid of
21 essentially any molecular weight in a rapid, high-yield
22 manner.

23 The nucleic acid aggregate binds to the matrix suspension
24 as a result of contacting the sample containing the aggregated
25 nucleic acid with the matrix suspension. The matrix
26 suspension may be contained in a column, and thus, as the
27 sample is passed throughout the matrix suspension, the nucleic
28 acid becomes bound to the hydrophilic non-net-positively

1 charged surface of the matrix. The matrix suspension also may
2 be contained in a conventional tube, dish or well, such that
3 the sample is mixed with the matrix, rather than passed
4 through it. Alternatively, the sample may be applied to a
5 matrix which is attached to a surface, e.g., mesh, beads, a
6 plate, a column, or the like. In this embodiment, the sample
7 is passed over the matrix for the nucleic acid to bind.

8 Some examples of matrix formats useful according to the
9 invention include passing the aggregated nucleic acid through
10 a column containing a matrix suspension, contacting the
11 aggregated nucleic acid with free fibers in suspension,
12 contacting the nucleic acid with fibers that are attached to a
13 support, whether the support be a mesh, a bead, a plate well,
14 or the like.

15 Where the matrix is cellulose, the invention contemplates
16 a matrix suspension of cellulose rather than a packed
17 cellulose column, as it has been discovered that a cellulose
18 matrix suspension is superior to a packed cellulose column in
19 binding nucleic acid.

20 However, non-cellulose matrices according to the
21 invention may be utilized in a packed matrix column if the
22 efficiency of binding is not at least two-fold better in
23 suspension than in a packed column format.

24 Once the nucleic acid binds to the matrix, digested or
25 solubilized proteins and salts do not bind and thus are
26 separated from nucleic acid in that they flow through the
27 matrix. The bound nucleic acid is eluted from the matrices
28 and recovered in a substantially pure and concentrated state,

1 suitable for direct use.

2 Solid hydrophilic organic polymers that constitute a
3 matrix useful according to the invention fall within the
4 definition provided hereinabove. A matrix according to the
5 invention will include any solid, hydrophilic organic matrix
6 without an effective positive charge that reversibly binds
7 nucleic acid substantially by weak forces such as Van der
8 Waals interactions and not by electrostatic interactions,
9 affinity binding, or physical trapping. Preferably, the
10 matrix is essentially neutral, i.e., without any positive or
11 negative charge.

12 The term "solid matrix", as used herein, encompasses a
13 polymer that is substantially insoluble in water and alcohol
14 at less than about 50 degrees centigrade. Preferably, a solid
15 matrix is in particulate form, with the particles being in the
16 micro-meter range (preferably, 5-500 μ meters) or the milli-
17 meter range (preferably, 0.1-10 millimeters); or is in fibrous
18 form with the fibers being micro-meter in diameter and of any
19 desired length.

20 The term "polymer" includes matrices made from repeating
21 units of two or more monomer repeats. As used herein,
22 "polymer" also includes homopolymers and heteropolymers, a
23 "homopolymer" being defined as a polymer consisting
24 essentially of repeating units of identical monomers, and a
25 "heteropolymer" being defined as a polymer consisting
26 essentially of two or more monomers which are not identical,
27 the monomers being repeated in a given order or randomly. A
28 "mixed polymer" is defined herein as including two or more

1 homopolymers or heteropolymers, or a combination of a
2 homopolymer and a heteropolymer.

3 Exemplary monomer materials include acrylonitrile,
4 acrylene, caprolactam, chloroprene, dichloroethene, ethylene,
5 isoprene, propylene, tetrafluoroethene, vinyl chloride,
6 vinylidene fluoride, acrylamide, amino acids, diisocyanate,
7 divinylbenzene, ethylene glycol, formaldehyde, glycol, methyl
8 methacrylate, styrene, sugars, terephthalic acid. Additional
9 exemplary polymer materials include but are not limited to
10 polysaccharides including cellulose, rayon, cellulose acetate,
11 cellulose triacetate, chitin and agarose; protein/polypeptides
12 including leather, silk and wool; synthetic gels including
13 polyacrylamide, hydrogel (i.e., copolymer of poly(vinyl
14 alcohol) and collagen); synthetic fibers including polyamides
15 (nylon), polyesters, polyacrylonitrile (acrylic), polyurethane
16 (spandex); and synthetic plastics including polycarbonate,
17 phenol-formaldehyde resins, polysulfide, poly(vinyl butyryl),
18 poly(vinyl chloride), poly(vinylidene chloride),
19 poly(ethylene), and polystyrene.

20 The present invention does not utilize ion-exchange
21 procedures, affinity binding, density gradients,
22 aqueous/organic phase separation, or physical trapping to
23 separate the nucleic acids from other cellular or tissue
24 components. Thus, subsequent removal of large amounts of
25 salt, as is present in samples having been prepared by such
26 methods, or further purification of nucleic acids, as is
27 necessary for samples having been prepared using gradients, is
28 not necessary. Moreover, the method of the invention is fast

1 and the yield of recovery for large sizes of nucleic acids is
2 superior to other methods of the prior art.

3 The invention features methods and devices for the
4 efficient and quantitative recovery of relatively small
5 amounts of nucleic acid from single or plural samples by
6 binding to a matrix having the characteristics described
7 herein.

8 The nucleic acid to be isolated can be present in any
9 type of biological sample, and will generally be a sample of
10 medical, veterinary, forensic, environmental, nutritional,
11 scientific or industrial significance. Human and animal
12 specimens and body fluids particularly can be assayed by the
13 present method, providing that they contain cells, or
14 particles, e.g., virions, from which nucleic acid can be
15 prepared. Preferred sources include blood, sperm, any
16 mammalian tissue, milk, urine, cerebrospinal fluid, sputum,
17 fecal matter, and lung aspirates, all of which may have been
18 collected as is or previously adsorbed onto a fluid collection
19 device such as a swab; in addition, buccal cells, throat
20 swabs, genital swabs and exudates, rectal swabs, and
21 nasopharyngeal aspirates.

22 The invention allows for simultaneous recovery of
23 exceedingly small amounts of nucleic acid from, e.g., hundreds
24 of a type of sample in a quantitative manner. Typically 70-
25 100%, and more likely at least 80%, 90% or most likely at
26 least 95%, of the nucleic acid that is present in a biological
27 sample may be recovered according to the invention, even when
28 the sample contains such small amounts as less than 1 ng of

1 nucleic acid. For example, as much as 0.3-0.4 μ g of genomic
2 DNA may be recovered from a 15 μ l dried blood spot according
3 to the invention. Because the invention allows for recovery
4 of the isolated nucleic acid into a relatively small volume of
5 liquid, e.g., smaller than 500 μ l, 250 μ l, 100 μ l, and even as
6 small as 5-10 μ l, the invention avoids the risk of loss of the
7 recovered nucleic acid encountered in concentrating a sample
8 from a relatively large volume.

9 The nucleic acid may be isolated or concentrated
10 according to the invention from an impure, partially pure, or
11 a pure sample. The purity of the original sample is not
12 critical to the inventive methods, as nucleic acid may be
13 isolated from even grossly impure samples according to the
14 invention. For example, nucleic acid may be removed from an
15 impure sample of a biological fluid such as blood, saliva, or
16 tissue. If a sample of higher purity is desired, the sample
17 may be treated according to any conventional means known to
18 those of skill in the art prior to undergoing isolation
19 according to the invention, e.g., the sample may be processed
20 so as to remove certain impurities such as insoluble materials
21 from an impure sample prior to nucleic acid isolation.

22 Methods of the invention may be performed on a biological
23 sample which has been deposited on any type of material,
24 provided the material itself does not form cross-linkages to
25 retain the nucleic acid irreversibly. Thus, the sample may be
26 contained within a material such as paper, textile, e.g., a
27 fragment of an article of clothing, agarose, or
28 polyacrylamide. One example of a sample and sample matrix

1 according to the invention is a drop of a body fluid, e.g.,
2 blood, which has been stored dry on a piece of Schleicher and
3 Schuell #903 paper, the paper routinely used nationwide for
4 the purpose of newborn screening.

5 For purposes of the following description of the
6 invention, recovery of nucleic acid in the form of genomic
7 DNA, plasmid DNA, or single-stranded DNA or RNA is described
8 in detail herein. However, it is to be understood that the
9 invention encompasses recovery of any form, whether
10 fragmented, circular, single stranded (RNA and some
11 bacteriophage and virus DNAs and RNAs) or chromosomal DNA.

12 The method of the invention is applied to isolation of
13 nucleic acid from a biological sample, as follows.

14 Tissues or cells that contain nucleic acids are suspended
15 in an extraction solution that contains a buffer system, a
16 detergent, and a chelating agent. The buffer system can be
17 any buffer, e.g., TrisHCl, sufficient to maintain pH values
18 from approximately 5.0 to approximately 10. The detergent can
19 be ionic or nonionic detergent, such as sodium dodecyl sulfate
20 (SDS) or octylglucoside, at a concentration sufficient to lyse
21 cells and denature proteins. A chelating agent, such as EDTA,
22 captures free divalent ions (Mg^{2+}) so that nucleic acids are
23 more soluble and protected from degradation by DNA-degrading
24 enzymes that require Mg^{2+} .

25 A protease may also be added to the extraction mixture to
26 digest proteins so that the nucleic acids can be easily
27 released from the cells and so that the proteins are degraded
28 to small peptides and become more soluble in solution. Any

1 non-specific or specific protease may be used, for example,
2 proteinase K, trypsin, chymotrypsin, or V8 protease.

3 Nucleic acid aggregates are next formed by precipitating
4 the nucleic acid. Structurally, nucleic acid possesses a
5 phosphodiester backbone that is negatively charged around
6 neutral pH. Nucleic acid becomes insoluble (i.e.,
7 precipitated) in the presence of salts and agents that can
8 reorganize its aqueous environment. Acetone, alcohols such as
9 ethanol and isopropanol, and soluble organic polymers, such as
10 polyethylene glycol (PEG) are examples of useful precipitants.
11 Co-precipitants, such as glycogen, also may be used to
12 facilitate the precipitation of nucleic acid present in only
13 small quantities; for example, in the form of
14 heteroaggregates. The presence of a co-precipitant is not
15 required according to the invention, but serves to increase
16 the efficiency of aggregate formation. In the presence of
17 monovalent salt, the charges in nucleic acids are neutralized
18 so that nucleic acid aggregates are formed and stabilized by
19 weak forces such as Van der Waals forces. Divalent salts such
20 as magnesium chloride or calcium chloride can also be used to
21 precipitate nucleic acids.

22 As indicated above, the matrix may be any matrix that
23 reversibly binds nucleic acid. In preferred embodiments
24 described in detail herein, cellulose, agarose powder, and
25 polyacrylamide are used as matrices. Where the matrix is
26 fibrous, the fibers may be mechanically broken to 1 to 2 mm
27 fibers. The matrix is washed to remove salts and other
28 contaminants. The matrix can either be stored dry or in an

1 aqueous solution at room temperature in a concentration
2 convenient for use. The matrix is in suspension and may be
3 used in a column format, for example, in a pipet tip, syringe,
4 or larger column containing a matrix suspension.

5 Alternatively, the matrix may be coated onto a well or an
6 inorganic microparticle 3-4 microns, such as a magnetic or
7 silicon bead, such that the nucleic acid binds to the matrix
8 coating on the particle. This embodiment is advantageous over
9 the isolation of nucleic acid via physical entrapment by
10 uncoated microparticles, as the purity and yield of the
11 nucleic acid is better.

12 Once the matrix is prepared, the matrix is added to the
13 suspension containing the nucleic acid before or after
14 aggregation, or the nucleic acid aggregate suspension is
15 allowed to bind to the matrix in the column. Unbound
16 materials, such as digested proteins, lipids, and other
17 unwanted cellular components are then separated from the bound
18 nucleic acid aggregates by retaining the nucleic acid/matrix
19 complexes in a column, filter, tube, or plate well. In one
20 embodiment, when the matrix is in a column format, the bound,
21 aggregated nucleic acids may be purified by washing the column
22 with a buffer to wash away the undesirable materials. Once
23 these materials are removed, the aggregated nucleic acid may
24 be recovered by eluting them from the matrix material after
25 dissolving the aggregates in water or low salt buffer. The
26 recovered nucleic acid is substantially pure, concentrated,
27 and suitable for immediate use in subsequent experiments.

28 The invention is better illustrated with respect to the

1 following examples. These examples are meant to be
2 illustrative of carrying out the invention, and not to be
3 limiting with respect to the spirit and scope of the invention
4 and the claims.

5 EXAMPLE 1

6 Preparation of Cellulose Matrix and Experimental Conditions.

7 Materials and reagents used in this Example and in
8 general (unless otherwise specified) are as follows:

- 9 1. TE buffer (10 mM TrisHCl, 1 mM EDTA, pH 8).
- 10 2. Matrix solution: 50 mM TrisHCl, pH 8.0, 10 mM EDTA.
- 11 3. Extraction buffer: 1% SDS, 50 mM TrisHCl pH 8, 10 mM
12 EDTA, 150 mM NaCl, 20 ug glycogen/ml, 50 ug/ml proteinase k.
- 13 4. Washing solution; 60% ethanol in buffer which
14 contains 200 mM NaCl, 50 mM TrisHCl, 5 mM EDTA.
- 15 5. Elution buffer: 5 mM TrisHCl pH 9, 0.5 mM EDTA.
- 16 6. 40% PEG in water.
- 17 7. 20% PEG in 2.5 M NaCl.
- 18 8. 5 M NaCl.
- 19 9. GEDTA: 120 g of GuSCN in 100 ml of 0.2 M EDTA at pH
20 8.
- 21 10. Isopropanol (precipitant).
- 22 11. Co-precipitant: glycogen (5 ug/ul).
- 23 12. TBE buffer: 45 mM Tris-Boric acid and 1 mM EDTA.
- 24 13. Whatman cellulose: 3MM paper is cut and dissociated
25 into fibers with an average length of 1-2 mm (length is not
26 critical). This is the matrix used in the following
27 experiments unless otherwise specified.

1 14. Sigma cellulose: Fibrous medium (C6288=CF-11), washed
2 to remove small particles and resuspended in TE buffer (10 mM
3 TrisHCl, 1 mM EDTA, pH 8) at 10 mg/ml.

4 15. Control DNA: Sigma, Human placenta DNA (D7011),
5 dissolved in TE buffer at 0.26 ug/ul.

6 16. Whole blood: anticoagulated in EDTA. Aliquotted for
7 use as 250-ul-samples and stored at -70°C.

8 17. Matrix collection device: AP-200 (P-1000) tip with a
9 barrier made up of a folded Kimwipe paper (1/4" disk for P-
10 200, 10x10 mm for P-1000 tip). Such a device is not able to
11 retain nucleic acid under conditions to be described.

12 18. All experiments are carried out at room temperature
13 unless otherwise specified.

14 19. Centrifugation is performed in a standard Ependorf-
15 type microcentrifuge.

16 Matrix is prepared by the following method unless
17 otherwise specified. Filter paper consisting essentially of
18 cellulose (0.25 g of Whatman 3 MM paper) is cut into < 4 mm²
19 pieces and suspended in about 10 ml of matrix solution,
20 followed by vigorous vortexing until the paper becomes
21 fibrous. The suspension is filtered through a membrane with
22 0.45 micron or larger pores to remove liquid and the fibers
23 are recovered from the filter. The wet fibers are then
24 subjected to the procedure of cutting-vortexing-filtration
25 twice so that all cellulose fibers are dissociated. The
26 fibrous matrix thus formed is stored in either 50 ml matrix
27 solution or 50 ml washing solution at room temperature (5
28 mg/ml). When stored in this manner, the fiber suspension is

1 free to pass through a pipet with 2 mm opening. The volume
2 that the 0.25g matrix occupies after settling is equivalent to
3 10 ml-12.5 ml.

4 Matrix-collection device: A cylinder or conical-shaped
5 container that is open at both ends to liquid movement and fit
6 with a barrier on one end to prevent solid materials from
7 passing through.

8

9

EXAMPLE 2

10 Isolating DNA from Dried Blood Spots on Filter Paper

11 Using Cellulose Matrix.

12 Solutions and reagents are the same as those used in
13 Example 1 unless otherwise specified. In addition, 1 M MgCl₂
14 and chelating resin are used. Matrix suspensions are prepared
15 as in Example 1 unless otherwise specified.

16 4 full circles (15 mm diameter) of dried blood spots are
17 removed from S&S 903 filter papers. Two of them are immersed
18 in 5 ml of extraction buffer (Sample 1) and the other two are
19 placed in 5 ml of the same buffer plus 0.1 g chelating resin
20 (Sample 2). The samples are incubated at 56°C for about 2
21 hours to digest proteins by proteinase K.

22 a. Phenol-extraction and ethanol precipitation (control
23 method):

24 1. 500 µl of Sample 1 (designated 1-0) and 500 µl of
25 Sample 2 (designated 2-0) are extracted with 500 µl of
26 phenol:chloroform twice.

27 2. Glycogen is added to 20 µg/ml and NaCl is added to

1 0.1 M.

2 3. 1 ml ethanol is mixed with the extracted samples to
3 precipitate nucleic acids at -20°C for 2 hours.

4 4. Precipitated nucleic acids are collected by
5 centrifugation for 15 min at 12 k rpm and finally dissolved in
6 50 µl of water.

7 B. Matrix method (method of the invention):

8 1. 4 aliquots of 500 µl (1-1 to 1-4) are retrieved from
9 Sample 1, and the same for Sample 2 (2-1 to 2-4). NaCl was
10 added to 0.1 M in each.

11 2. The samples are mixed with co-precipitants: glycogen
12 (10 µg in 1-1, 1-2, 2-1, 2-2) and Mg²⁺ (final concentration 20
13 mM in 1-1, 1-3, 2-1 and 2-3).

14 3. Isopropanol (650 µl) is mixed with each sample and
15 the mixtures are incubated at room temperature for 20 min to
16 precipitate nucleic acids.

17 4. Precipitated nucleic acids are loaded onto pre-
18 equilibrated matrix (5 mg matrix each) connected to a vacuum
19 manifold unit.

20 5. The matrix are washed twice with 1 ml washing
21 solution under vacuum and dried by centrifugation at 5 k rpm
22 for 1 min.

23 6. 50 µl of elution buffer is added to each dried
24 matrix to dissolve nucleic acids at room temperature for 5
25 min. The nucleic acids in a sample are recovered into a 1.5
26 ml tube by centrifuging the tubes at 7 k rpm for 2 min.

27 5 µl of each control sample (1-0 and 2-0) and the samples
28 purified by columns are separated in 1% agarose gel containing

1 0.5 ug/ml of ethidium bromide by a standard method.

2 The same amount of nucleic acid (mainly DNA) is recovered
3 by the matrix method as by the control method, based on gel
4 electrophoresis analysis. Samples treated with chelating
5 resins contained DNAs of relatively large molecular weights.

6 DNA isolated from filter paper by the nucleic acid
7 isolation method described herein is consistently found to be
8 compatible with DNA modifying enzymes. The DNA thus-isolated
9 is also digestible by restriction enzymes such as ScrF1, and
10 can be used for polymerase chain reaction (PCR).

11 Thus, high yield isolation of nucleic acid from small
12 quantities of clinical samples may easily be achieved using
13 the method of the invention. Because high speed
14 centrifugation ($>10,000$ rpm) is not required, the method can
15 be easily automated. The total isolation time for solubilized
16 nucleic acids may be shorter than 30 min.

17 EXAMPLE 3

18 Isolation of Plasmid DNA Using Cellulose Matrix.

19 Reagents, matrix and columns are the same as in Example 1
20 unless otherwise specified. In addition, a plasmid isolation
21 kit (Wizard Kit) from Promega was used for comparison. LB
22 media used in this example contained 1% tryptone, 0.5% yeast
23 extract and 1% NaCl in water.

24 Source of double-stranded plasmid DNA: E. coli cells
25 harboring pBluescript plasmid (Stratagene) are grown overnight
26 in LB media containing ampicillin (100 µg/ml).

27 Source of single-stranded plasmid DNA: E. coli cells
28 harboring pBluescript plasmid (Stratagene) are infected by

1 M13K07 helper phages (NEB) to generate single-stranded plasmid
2 DNA. The cells are grown overnight at 37°C in LB media
3 containing ampicillin (100 µg/ml) and kanamycin (70 µg/ml).

4 To purify double-stranded plasmid DNA, the following
5 steps are performed:

6 1. An overnight culture is split into 6 aliquots of 1-
7 ml samples (a-1 to a-6).

8 2. Crude plasmid DNA is obtained by alkaline lysis
9 method with reagents in the Wizard plasmid isolation kit,
10 following instruction from the manufacturer.

11 3. DNAs in samples a-1 to a-3 are further purified with
12 silica powder provided in the same Wizard kit. Each DNA
13 sample is finally recovered in 50 µl of water.

14 4. Other crude DNA samples (a-4 to a-6) are mixed with
15 750 µl of isopropanol and incubated for 20 min at room
16 temperature.

17 5. Precipitated DNAs are mixed with 5 mg matrix and
18 prepared as described in Example 1.

19 6. Washing and DNA recovering are the same as described
20 in Example 1 and 2. DNA in each column is eluted in 50 µl of
21 elution buffer.

22 To purify single stranded plasmid DNA, the following
23 steps are performed:

24 1. 8 clear supernatants of 1 ml (B-1 to B-8) are
25 recovered from a helper phage-infected culture after removal
26 of cells by centrifugation.

27 2. 250 µl of PEG solution (20% polyethylene glycol-8000
28 plus 2.5 M NaCl) is mixed with each of the 8 samples. The

1 solutions are kept at room temperature for 15 min.

2 3. Precipitated phage particles are harvested by
3 centrifugation at 10k rpm for 5 min at room temperature.

4 4. After complete removal of the liquid, 500 μ l of
5 extraction buffer is added to each pellet and incubated at
6 56°C for 40 min to release DNA. NaCl is then added to 0.2 M.

7 5. 4 samples (B-1 to B-4) are subjected to
8 phenol:chloroform extraction twice followed by ethanol
9 precipitation as described in Example 2. The DNA pellets are
10 dissolved in 50 μ l of TE buffer.

11 6. The other 4 samples (B-5 to B-8) are mixed with 10
12 μ g glycogen and 625 μ l of isopropanol, followed by
13 purification with matrix as described in Example 1. Each DNA
14 sample for recovery is dissolved in 50 μ l of elution buffer.

15 5 μ l of each purified DNA is separated in a 1% agarose
16 gel containing 0.5 μ g/ml ethidium bromide for analysis.

17 The results demonstrate that similar amounts of double-
18 stranded plasmid DNAs are isolated with cellulose matrix as
19 with a silica matrix. Single-stranded plasmid DNAs are also
20 isolated, although the amount of DNA isolated by the cellulose
21 matrix method is slightly less than that isolated after phenol
22 extraction and ethanol precipitation. DNAs isolated by the
23 cellulose matrix method are sequenced as efficiently as DNAs
24 isolated by phenol extraction and ethanol precipitation
25 method. Plasmid isolation by this method of the invention
26 eliminates the requirement for chaotropic agents and minimizes
27 the use of high speed centrifugation.

EXAMPLE 4

RNA Isolation Using Cellulose Matrix.

Solutions and reagents are the same as in Example 1 unless otherwise specified.

RNA may be isolated by the following steps:

1. 1 g of fresh and soft plant leaves is ground in 5 ml of TRIzol (Life Technologies) to release nucleic acid.

2. The homogenate is separated by centrifugation and two clear supernatants of 1 ml each are collected. Each of the supernatants is mixed with 600 μ l of chloroform. 750 μ l of the aqueous solution is recovered from each tube after centrifugation and is placed in a clean tube.

3. 750 μ l of isopropanol is mixed with each solution and the resulting solutions are kept at room temperature for 20 min to precipitate the nucleic acids.

4. Nucleic acid in one tube (A) is harvested by centrifugation (12,000 rpm) for 15 min at room temperature. The pellet is dissolved in 75 μ l of water after removal of liquid and drying the pellet. 25 μ l of the sample is diluted 1:1 with 25 μ l of water and the resulting solution is designated as A1 and the remaining 50 μ l A2.

5. Nucleic acid precipitated by isopropanol in the other tube (B) is divided to 500 μ l (B1) and 1000 μ l (B2). Nucleic acid in the two solutions are subjected to matrix purification as described in Example 1 and 2. The nucleic acid from each sample is dissolved for recovery in 50 μ l water.

6. 5 μ l of each sample (A1, A2, B1, B2) may be

1 subjected to agarose gel electrophoresis (1.2%, 0.5 µg/ml
2 ethidium bromide).

3 7. 400 µl water is added to each sample and the
4 resulting solutions are examined by a UV spectrophotometer.

5 RNAs of small (tRNA) and large (rRNA) sizes are isolated
6 with the cellulose matrix, and DNA of very high molecular
7 weights is also present. The band patterns of the RNAs
8 obtained by the two methods are identical. Sample A2
9 contained the largest amount (114 µg) of nucleic acids (mainly
10 RNA) and sample A1 contains approximately half (62 µg) as much
11 as A2. Sample B1 has nearly the same amount (53 µg) of
12 nucleic acids as A1. Sample B2 recovers approximately 81 µg.

13 In this example, A1 and A2 are prepared by the complete
14 TRIzol method, and B1 and B2 by a modified method in which
15 the cellulose matrix method is used to replace high speed
16 centrifugation. Thus, the quality of the RNAs are expected to
17 be as good as that isolated by the complete TRIzol method.
18 The column procedure may be more reliable for isolating small
19 quantities of nucleic acids (<20 µg) because pellet formation
20 is not required.

21 EXAMPLE 5

22 Demonstration of Cellulose for DNA Isolation and Concentration 23 From Liquid Whole Blood Samples Using the Suspension Format.

24 SETUP

25	Samples	1	2	3	4	5	6	7	8
26	Buffer								
27	Extraction buffer:	+	+	+	+	-	-	-	-

1	GEDTA:	-	-	-	-	+	+	+	+
2	Matrix								
3	Whatman cellulose:	-	-	+	+	-	-	+	+

4 Procedure A: (Using SDS/proteinase-K-containing Extraction
5 buffer for nucleic acid solubilization)

6 1. Add 250 ul Extraction buffer and 12.5 ug proteinase K
7 to each 250 ul whole blood sample, total 4 samples (#1-#4).

8 2. The samples are incubated at 56°C for 1.5 hours.

9 3. Add 250 ul of 40% PEG and 12.5 ul of 5 M NaCl to each
10 sample; mix the samples for about 5 min.

11 4. Centrifuge the samples for 2 min at 2 k rpm and
12 recover the supernatant for each sample.

13 5. Add 5 mg Whatman cellulose to Samples 3 and 4 and add
14 250 ul 5 M NaCl to all the 4 samples; mix the samples for
15 about 5 min.

16 6. Centrifuge the samples for 2 min at 2 k rpm; save
17 pellets 1,2,3,4 and recover each supernatant; designate
18 supernatants as 1',2',3',4' respectively.

19 7. Centrifuge samples 1'-4' for about 5 min at 14 k rpm
20 to collect any precipitant; discard supernatant.

21 8. Add 1 ml washing solution to each of the samples #1,#2
22 and 1'-4'; after a gentle mixing, centrifuge the 6 samples
23 together as Step 7; the pellets are air-dried and each sample
24 is dissolved in 100 ul of elution buffer.

25 9. Cellulose fibers in samples 3 and 4, which look
26 reddish, are collected in independent matrix-collection
27 devices, washed twice with total 2 ml washing solution.

1 10. The collected cellulose is dried by spinning at 5 k
2 rpm for 2 min and DNA of each sample is eluted twice with a
3 total of 50 ul elution buffer. The resulted colorless DNA
4 solutions are finally adjusted to 100 ul for each.

5 11. 10 ul of each sample is analyzed in a 1% agarose gel.

6 Procedure B: (Using chaotrope-containing solution for nucleic
7 acid solubilization)

8 1. Add 250 ul GEDTA to each 250 ul whole blood, total 4
9 samples (#5-#8); mix the samples for 5 min.

10 2. Add 250 ul of isopropanol to each sample and 5 mg
11 Whatman cellulose to #7 and #8, mix the samples for 5 min.

12 3. Centrifuge the samples for 2 min at 2 k rpm; save the
13 pellets (5-8) and recover the supernatant (5'-8').

14 4. Centrifuge all the supernatants at 14 k rpm for 5 min;
15 air-dry the pellets and dissolve each pellet in 100 ul of
16 elution buffer.

17 6. Add 250 ul of water and 250 ul of GEDTA to cellulose
18 in #7 and #8, which look red; after mixing for 2-3 min, add
19 250 ul of isopropanol to each and mix again for another 2-3
20 min.

21 7. The cellulose fibers in sample #7 and #8 are collected
22 by a matrix collection device separately.

23 8. The fibers in a matrix collection device are washed
24 twice with total 2 ml washing solution; the fibers are then
25 dried by spinning at 5 k rpm for 2 min.

26 9. DNA associated with the cellulose fibers is eluted
27 twice in a total of 50 ul elution buffer. Each colorless DNA

1 solution is finally adjusted to 100 ul.

2 10. 10 ul of each sample is analyzed in a 1% agarose gel.

3 The results were as follows.

4 1. For samples that do not contact the cellulose, the
5 majority of the DNA remains in the first supernatant (1 and 2
6 vs. 1' and 2'; 5 and 6 vs. 5' and 6').

7 2. For samples that contact the cellulose, a significant
8 amount of DNA is in the pellet fraction after low speed
9 spinning (3,4,7,8), indicating that the precipitated nucleic
10 acid is associated with cellulose in suspension. We attribute
11 the nucleic acid observed in the supernatant fraction
12 (3',4',7',8') to fiber-DNA complexes that are carried over.

13 3. Procedure B is simpler than Procedure A. But GEDTA is
14 a more hazardous solution. A260nm/A280nm is about 1.8 for
15 sample #7 and #8.

16 4. Less total nucleic acid is recovered with Procedure A,
17 likely to be due to incomplete cell lysis under the described
18 conditions. Higher yields are observed when increased SDS
19 concentration is used for more diluted samples.

20 The conclusions were as follows.

21 1. Re-extracting the nucleic acid is a necessary step for
22 removal of pigmented and other contaminants.

23 2. Suspension format is a convenient way for the re-
24 extraction step.

25 3. Nucleic acids bind to cellulose efficiently in
26 suspension.

4. Nucleic acid can be isolated in a concentrated form:
nucleic acid in >0.25 ml of blood can be concentrated to a
final volume of <50 ul.

4 5. The isolated DNA is pure: eluted DNA is colorless with
5 little protein contamination.

6 EXAMPLE 6

7 DNA isolation Using Agarose Matrix.

8 Solutions and reagents were the same as in Example 1
9 unless other specified. Dry agarose powder (FMC, Type LE) is
10 suspended in water at room temperature at a concentration of
11 approximately 5 mg/ml. Matrix is washed with water and matrix
12 solution before they are used for DNA isolation.

13 DNA sample preparation (1.75 μ g of Salmon Testes DNA for
14 each sample) and isolation procedures are the same as
15 described in Example 1 and 2. Isolated DNAs are analyzed by
16 agarose gel and optical density measured. About 92% of DNA
17 can be recovered by the agarose matrix, compared to those
18 recovered by ethanol precipitation when the same amounts of
19 DNA are used.

20 EXAMPLE 7

21 DNA Isolation Using Synthetic Fiber Matrix.

Solutions and reagents are the same as in Example 1 unless otherwise specified. Synthetic cotton from a cosmetic puffball (purchased from a local department store) is cut to short fragments (1-2 mm).

26 Sample preparation, DNA isolation and analysis are the

1 same as described in Example 8. About 50% of DNA is recovered
2 with this matrix, compared to those recovered by ethanol
3 precipitation when the same amounts of DNA were used. This
4 relative low yield of recovery is expected due to the lower
5 hydrophobicity of this matrix compared to a cellulose matrix.

6 EXAMPLE 8

7 DNA Isolation Using Polyacrylamide Matrix.

8 Solutions and reagents are the same as in Example 1
9 unless otherwise specified. 7.5 ml of 30%
10 acrylamide/bisacrylamide solution and 7.5 ml of water were
11 mixed. a 15% polyacrylamide gel was formed and is broken into
12 fine particles (0.5-1.5 mm) mechanically. The suspension is
13 washed with water extensively until soluble materials and
14 unpolymerized acrylamide are removed.

15 Sample preparation and subsequent purification procedures
16 are essentially the same as described in Example 2. DNA is
17 recovered in basically the same yield by the polyacrylamide
18 matrix as by cellulose matrix.

19 A summary of recovery of nucleic acids, relative to a
20 100% recovery of nucleic acid using the phenol or silica
21 isolation methods described hereinabove, is provided in Table
22 I.

23 In Table I, the following key is used. Phenol refers to
24 phenol/chloroform extraction followed by ethanol
25 precipitation; Silica refers to a silica glass powder matrix
26 for DNA purification; CF refers to a cellulose fiber matrix
27 for DNA purification; Agarose refers to agarose powder matrix

1 for DNA purification; Synthetic refers to a synthetic cotton
 2 fiber matrix for DNA purification; PAG refers to a
 3 polyacrylamide gel suspension matrix for DNA purification.

4 In Table 1, the DNA referred to is as follows. Lambda:
 5 lambda DNA; Salmon: salmon testes DNA; DBS: dried blood spot
 6 DNA; DSP: double stranded plasmid DNA; SSP: single stranded
 7 plasmid DNA; Plant: plant RNA. In addition, each number
 8 refers to percentage of recovery, control is 100%; NA refers
 9 to not applicable, or not available; + refers to the same or
 10 nearly the same as controls.

1 TABLE I

2 -----

3 <u>Method</u>	<u>Form or source of nucleic acids</u>					
4	<u>Lambda</u>	<u>Salmon</u>	<u>DBS</u>	<u>DSP</u>	<u>SSP</u>	
5 <u>Plant</u>						
6 Phenol	100	100	100	NA	100	
7 100						
8 Silica	NA	NA	NA	100	NA	NA
9 CF	100	99	+	+	70-80	+
10 Agarose	NA	92	NA	NA	NA	NA
11 Synthetic	NA	50	NA	NA	NA	NA
12 PAG	NA	NA	+	NA	NA	NA

13 -----

14

15 EXAMPLE 9

16 DNA Isolation Using Cellulose Coated Particles.

17 Solutions and reagents are the same as in Example 1
 18 unless otherwise specified. The nucleic acid is first

1 solubilized and then aggregated by precipitation. Cellulose-
2 coated particles, e.g., magnetic beads, are added to the
3 aggregating buffer containing aggregated nucleic acid and the
4 aggregated nucleic acid is allowed to contact the cellulose
5 fibers on the beads for 2 or 3 minutes. The cellulose-coated
6 particles and associated nucleic acids are removed from the
7 solution by means commonly known in the art; in the case of
8 magnetic beads, a magnetic field is applied to draw the
9 nucleic acid away from the solution, which is then removed.
10 The magnetic field is then released. A wash solution is
11 applied and a magnetic field is applied. The magnetic field
12 is then released again. Elution of DNA from the beads is
13 accomplished by adding an aqueous buffer, applying the
14 magnetic field, and then removing supernatant-containing DNA.

16 Apparatuses of the Invention

17 As illustrated in Fig. 1, an apparatus of the invention
18 will include plural housings 100, 100' and a planar surface
19 support 104 for convenient simultaneous handling of the plural
20 housings. Each housing 100, 100' possesses an inlet 101, 101'
21 and an outlet 103, 103', and defines a flowpath 105, 105' for
22 flow of liquid therebetween. Housings 100, 100' contain
23 matrix 102, 102', as defined and described herein. The plural
24 housings and surface support 104 may be an integral unit, or
25 the housings may be separate from and adapted to fit into the
26 support 104. The housings may also include barrier means 106,
27 106' near the outlet end 103, 103', which barrier means serves
28 to prevent matrix from exiting the outlet of the housing.

1 The support 104 may be a plate or tray containing holes
2 into which the housings fit, or it may be a simple wire or
3 plastic rack. The apparatus may optionally include a
4 collection surface 107 which is positioned beneath the support
5 surface 104. The collection surface is also a planar surface
6 which includes sample collection sites 108, 108'. Sites 108,
7 108' may be simple indentations on the surface of a plastic
8 plate or they may be cups or tubes, e.g., microfuge tubes,
9 which fit into the plate. Collections sites 108, 108' may be
10 water insoluble, such as plastic, for collection of liquid
11 flowthrough from the housing, or they may be absorbent pieces
12 of filter paper for absorbing flowthrough. Sites 108, 108'
13 are for collecting liquid that flows through the housing,
14 whether it be matrix washings of unwanted material or eluted
15 nucleic acid. During operation, the apparatus may include two
16 collection surfaces of the format of surface 107, a first
17 collection surface 106 for collection of unwanted materials
18 which flow through the housings, and the second collection
19 surface 107 for collection of eluted nucleic acid. The
20 collection surface 107 may lie beneath surface 104; however,
21 optimally, surface 106 will fit snugly within the edges of
22 surface 104. In the embodiment of the invention shown in Fig.
23 1, flow through may be collected using gravity flow or by
24 centrifugation of the entire apparatus, or by pressure applied
25 from top of the housing.

26 Alternatively, as shown in Fig. 2, the apparatus may
27 include dish 109, which may include means for connecting 110
28 the apparatus to a vacuum source to assist in washing the

1 matrix. a vacuum source may be connected to the vacuum
2 connecting means 110 and a vacuum applied to suck excess
3 solutions from the column. Vacuum connecting means 110 may
4 include a connector such as a compression fitting, ferrule,
5 coupling, or other structure known in the art capable of
6 accepting and holding a vacuum. Although gravity flow may be
7 used to pull liquid through the housing, use of a vacuum unit
8 expedites the method of the invention.

9 The apparatus shown in Fig. 2 also may be combined with
10 the apparatus shown in Fig. 1. That is, surface support 104,
11 containing plural housings 100, 100', and collection tray 107
12 may be used along with dish 109 such that the collection tray
13 107 fits snugly within dish 109. When vacuum is applied to
14 the apparatus via connecting means 110, the vacuum pulls
15 liquid through the housing, matrix, outlet, and onto the
16 collection sites 108, 108'.

17 In operation, plural biological samples in liquid form
18 are applied to the inlet 101 of the plural housings, whereupon
19 each sample flows along flow path 105 into and through the
20 matrix 102. Contact between nucleic acid in the sample and
21 the matrix results in binding of nucleic acid to the matrix.
22 Nucleic acid is thus retained, while unwanted components of
23 the biological sample flow through the matrix and screen 106,
24 and exit via outlet 103. The matrix may be washed prior to,
25 during, or after nucleic acid binding, if desired.

26 After binding, which may take no longer than a few
27 minutes, or the time interval in which the liquid sample flows
28 through the matrix, bound nucleic acid is eluted from plural

1 matrices simultaneously by dispensing elution buffer into the
2 plural housings and fitting the support plate 104 over the
3 sample collection tray 107. Centrifugation and gravity may be
4 used to pull the elution buffer through the matrix (Fig. 1).
5 Alternatively, a vacuum source may be connected to dish 109
6 and support plate 104 may be placed over dish 109 to expedite
7 flowthrough (Fig. 2). If desired, support plate 104,
8 collection plate 107 and dish 109 may be sandwiched together
9 for simultaneous isolation of plural nucleic acid samples. If
10 desired, the unit can be modified so that liquid can flow
11 through the housing under pressure applied to 101 or 101', and
12 samples are collected via 108, 108'.

13 Another apparatus according to the invention for
14 isolation of substantially pure nucleic acid includes any
15 solid relatively inert organic surface such as, e.g., plastic,
16 an inorganic surface such as a metal surface, the surface
17 being coated with a matrix as described herein. An example of
18 preparation and use of a matrix-coated surface is provided
19 below.

20 For example, a polypropylene Column may be coated with
21 cellulose matrix as follows. 1 - 10 mg fibrous cellulose,
22 prepared as described herein is contacted with the activated
23 surface of the column; e.g., cellulose bound irreversibly to
24 the plastic. The amount of any type of matrix-coated on the
25 column will be that amount which is sufficient to bind nucleic
26 acid without a substantial amount of non-specific binding.
27 Non-specific binding is that binding which occurs in the
28 absence of matrix-coating. Non-specific binding is not

1 substantial when less than 5% of nucleic acid is bound in the
2 presence of matrix. The matrix will be irreversibly bound to
3 the support surface, i.e., such that it is not lost from the
4 surface upon elution of the nucleic acid from the support.

5 In use, a biological sample containing nucleic acid is
6 contacted with the matrix-coated support under conditions
7 which permit the nucleic acid to bind, as taught hereinabove.
8 The support may then be washed and the nucleic acid eluted, as
9 taught herein.

10

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OTHER EMBODIMENTS

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Other embodiments will be evident to those of skill in the art. Although the invention has been shown and described with respect to an illustrative embodiment thereof, it should be appreciated that the foregoing and various other changes, omissions, and additions in the form and detail thereof may be made without departing from the spirit and scope of the invention as delineated in the claims.

CLAIMS

1. A method of isolating nucleic acid in a substantially purified form, said method comprising the steps of:

a) contacting a biological sample comprising nucleic acid with a matrix under conditions which permit said nucleic acid in said sample to bind to said matrix, said matrix comprising a solid hydrophilic organic polymer without an effective positive charge; and

b) recovering said nucleic acid from said matrix.

2. The method of claim 1 wherein said conditions also permit said nucleic acid in said sample to aggregate.

3. The method of claim 1, said matrix comprising a matrix suspension.

4. The method of claim 1, further comprising the step of contacting said biological sample with a buffer under conditions sufficient to solubilize the nucleic acid.

5. The method of claim 1, wherein said conditions comprise incubating said sample in a nucleic acid precipitating solution.

6. The method of claim 1, further comprising the step, prior to step a) of aggregating said nucleic acid in said biological sample.

7. The method of claim 5, wherein said solution comprises a precipitating ingredient selected from the group consisting of organic solvents, soluble organic polymers and salts.
8. The method of claim 7, wherein the organic solvent may be any one of isopropanol, ethanol, and acetone.
9. The method of claim 7, wherein said organic polymer consists essentially of polyethylene glycol.
10. The method of claim 7, wherein said salt is one of Na⁺ and Li⁺.
11. The method of claim 1, wherein said matrix is selected from a group consisting of polysaccharides and polypeptides.
12. The method of claim 11, wherein said polysaccharides are selected from the group consisting of agarose and chitin.
13. The method of claim 3, said matrix comprising cellulose.
14. The method of claim 11, wherein said polypeptides are selected from the group consisting of leather, wool and silk.
15. The method of claim 1, wherein said matrix is selected from the group consisting of naturally occurring polysaccharides and polypeptides, synthetic hydrophilic polymers, and chemically modified polymers that are

hydrophobic polymers which become hydrophilic after chemical modification.

16. The method of claim 1, wherein said matrix is coated on a surface.

17. The method of claim 16, wherein said matrix is coated on an inorganic microparticle.

18. The method of claim 3, wherein said matrix suspension comprises cellulose-coated magnetic beads.

19. The method of claim 1, wherein said matrix is a synthetic gel.

20. The method of claim 19, wherein said synthetic gel is selected from the group consisting of polyacrylamide and hydrogel.

21. The method of claim 1, wherein said matrix is a synthetic fiber.

22. The method of claim 21, wherein said synthetic fiber is selected from the group consisting of polyamides, polyesters, polyacrylonitrile, and polyurethane.

23. The method of claim 1, wherein said matrix is a synthetic plastic.

24. The method of claim 23, wherein said synthetic plastic is selected from the group consisting of synthetic plastics including polycarbonate, phenol-formaldehyde resins, polysulfide, poly(vinyl butyryl), poly(vinyl chloride), poly(vinylidene chloride), poly(ethylene), and polystyrene.
25. The method of claim 21, wherein said synthetic fiber contains at least one polar group selected from the group consisting of hydroxyl, carboxyl, amino, and thiol.
26. The method of claim 18, wherein said synthetic plastic contains at least one polar group selected from the group consisting of hydroxyl, carboxyl, amino, and thiol.
27. A method of isolating nucleic acid in a substantially purified form, said method comprising the steps of:
- a) contacting a biological sample comprising nucleic acid with a matrix-coated support under conditions which permit said nucleic acid in said sample to bind to said matrix, said matrix comprising a solid hydrophilic organic polymer without an effective positive charge; and
 - b) recovering said nucleic acid from said matrix.
28. The method of claim 27, said surface comprising a surface selected from the group consisting essentially of a plate, a well, a column, and a microtiter dish.
29. An apparatus for isolating nucleic acid in a

substantially purified form from multiple biological samples simultaneously, said apparatus comprising:

a plurality of housings, wherein each said housing comprises an inlet and an outlet and defines a flowpath for flow of a sample therethrough, said flowpath comprising a matrix comprising a solid hydrophilic organic polymer without an effective positive charge, and

support means for holding said plurality of housings in place such that nucleic acid in said plural biological samples may be isolated simultaneously.

30. The apparatus of claim 29, wherein each housing of said plurality comprises a barrier means to allow flow of liquid along the flowpath and through the housing outlet, and to prevent the matrix from exiting the housing via the outlet.

31. The apparatus of claim 30, wherein each said housing comprises a lower portion leading to the outlet, and said barrier means is positioned within the lower portion of the housing.

32. The apparatus of claim 29, further comprising a connector for connecting said plurality of housings to a vacuum or pressure source.

33. The apparatus of claim 29, further comprising a collection tray for simultaneously collecting plural nucleic acid samples.

34. An apparatus for isolating nucleic acid in a substantially purified form from a biological sample, comprising;

a support surface containing an irreversibly bound matrix, said matrix comprising a solid hydrophilic organic polymer without an effective positive charge.

35. The apparatus of claim 34, said support surface comprising a cylindrical housing.

36. The apparatus of claim 34, said support surface comprising a multi-well plate.

37. The apparatus of claim 29 or 34, said matrix comprising cellulose.

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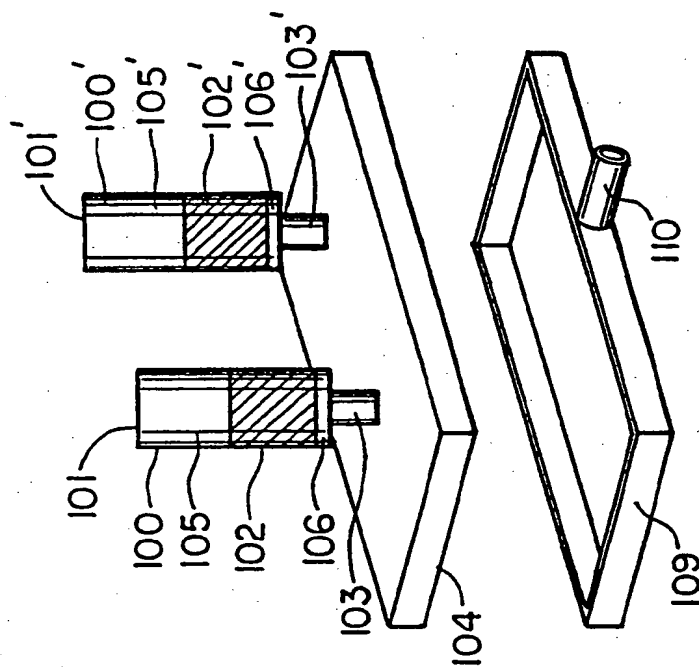


FIG. 2

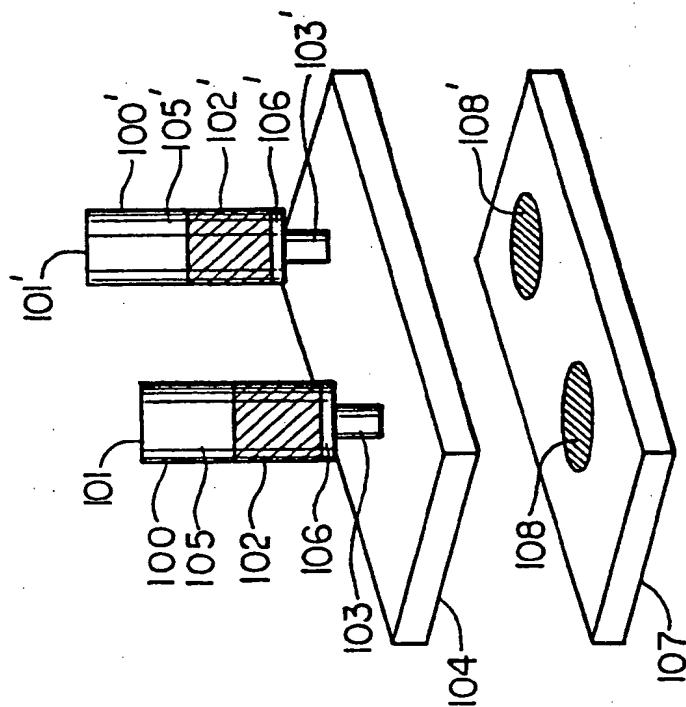


FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/13626

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 30/22; B01L 11/00; C12N 1/08; C07H 21/00

US CL : 422/70, 101; 435/270; 536/25.40, 25.41, 25.42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/70, 101; 435/270; 536/25.40, 25.41, 25.42

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
none

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,902,481 A (CLARK ET AL.) 20 February 1990, see entire document.	1-37
A	US 5,205,989 A (AYSTA) 27 April 1993, see entire document.	1-37
Y	US 5,417,923 A (BOJANIC ET AL.) 23 May 1995, see entire document.	1-37
A	US 5,219,528 A (CLARK) 15 June 1993, see entire document.	1-37
Y	US 5,108,704 A (BOWERS ET AL.) 28 April 1992, see entire document.	1-37
X	US 5,264,184 (AYSTA ET AL.) 23 November 1993, see entire document.	1
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Y		2-37

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

15 NOVEMBER 1996

Date of mailing of the international search report

27 NOV 1996

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/13626

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,160,803 A (POTTS) 10 July 1979, see entire document.	1-37
Y	US 5,217,593 A (MACCONNELL) 08 June 1993, see entire document.	1-29
Y	US 4,923,978 A (MCCORMICK) 08 May 1990, see entire document.	1-29
Y	US 5,346,994 A (CHOMCZYNSKI) 13 September 1994, see entire document.	1-29
Y	US 5,187,083 (MULLIS) 16 February 1993, see entire document.	1-29
Y	US 5,234,824 (MULLIS) 10 October 1993, see entire document.	1-29
Y	SCHLEIF et al. Practical Methods in Molecular Biology. Springer-Verlag: New York, NY, 1981, only pages 93-111 supplied, see entire document.	1-29
Y	Proteinase K for Nucleic Acid Research. BMBiochemica. December 1984, Vol. 1, No. 5, Page 3, see column 1.	1-29
Y	BEIL et al. Isolation of DNA from Fungal Mycelia and Sclerotia Without Use of Density Gradient Ultracentrifugation. Analytical Biochem. 1986, Vol. 154, pages 21-25, see entire document.	1-29
Y	BEJI et al. A Rapid Chemical Procedure for Isolation and Purification of Chromosomal DNA from Gram-Negative Bacilli. Analytical Biochem. 1987, Vol. 162, pages 18-23, see entire document.	1-29

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